

**THE USE OF A POROUS IMPLANT TO DEVELOP AN OPTIMUM  
METHOD TO EXAMINE BONE GROWTH/REPAIR FOR BIODYNAMIC  
AND TOXICOLOGIC ANALYSIS**

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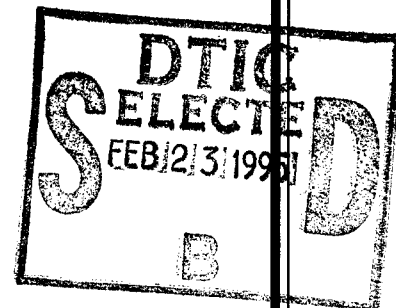
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## TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical paper has been reviewed and is approved for publication.

FOR THE DIRECTOR



THOMAS J. MOORE, Chief  
Biodynamics and Biocommunications Division  
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13. ABSTRACT (Maximum 200 words) <u>Introduction:</u> Bone repair in experimentally produced defects is regarded as very similar to the healing of bone after fractures. A cooperative effort within Armstrong Laboratory brought together a combination of resources to accurately assess bone growth and repair. The goal was development of standard procedures for use in determining the effects of biomechanical and toxicological testing. <u>Methods:</u> Simulated bone defects were surgically prepared in twelve rhesus monkeys. These defects were evaluated after one, four, or eight months using histomorphometry and scanning electron microscopy (SEM). <u>Results:</u> Histological evaluation of bone sections showed that bone removed from defect sites had not been replaced. There was no evidence of active bone formation. <u>Conclusion:</u> Healing of bone defects in this group of subjects was not successful under the experimental conditions used. Efforts to determine the reasons for this will not be possible due to new mission guidelines.				
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## PREFACE

The research described in this final report was completed under work unit #723122D4, Use of a Porous Implant to Develop an Optimum Method to Examine Bone Growth/Repair for Biodynamic and Toxicologic Analysis. It is the last program in this technical area reflecting changes in the laboratory mission. The investigation was a cooperative effort by the Vulnerability Assessment Branch (AL/CFBV), the Biochemistry Branch (OETB), and the Comparative Medicine Branch (OEVM), of the Armstrong Laboratory (AL) located at Wright-Patterson AFB, Ohio 45433-7901. The research was supported by the Laboratory Director's Fund Program.

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## INTRODUCTION

Bone has previously been analyzed in our laboratory using histomorphometric techniques employing fluorochrome dyes and general histology (AL/CFBV) or examined separately by scanning electron microscopy (SEM) with or without x-ray analysis by the Toxicology Division, Armstrong Laboratory (AL/OET). The present study uses both of these techniques in a single study. The availability of resources within the Armstrong Laboratory provided the capability to accomplish both these analytical techniques with a single sample.

Multiple fluorochrome labeling can be used to demonstrate both the amount of bone growth and rates of growth. Fluorescent bands mark the position of the mineralization front at the time a label is administered. Each subsequent band marks growth at a known point in time. Thus fluorochrome labeling enables bone growth to be detected and illustrated photographically. SEM provides researchers with information regarding changes in bone at the cellular level while x-ray analysis can be used to determine the elemental composition of bone without using wet chemistry methods. Using combined techniques, we proposed to demonstrate the growth of bone into a porous implant which has been suggested for use as a model for bone growth and repair. The combination of these procedures can be expected to yield a more complete picture of the processes involved in skeletal repair.

The healing process which occurs in experimentally produced defects in bone is similar to that which occurs during the healing of fractures (24). In the present study, a porous biomaterial was implanted into the cortical bone of the femur and trabecular bone of the vertebrae of rhesus monkeys in order to examine bone healing processes.

Ceramics have proven to be an ideal biomaterial for bone replacement. The resorption characteristics of ceramics determines the way they are used in orthopaedic surgery (26). Aluminum oxide ceramics are strong, inert and show good integration between bone tissue and the implant. Dense aluminum oxide ceramics have been used for total hip endoprostheses (13). Ceramic implants have also been made using various combinations of calcium aluminate or calcium and phosphates (6,15). The composition of calcium phosphate ceramics resembles the mineral phase of bone.

Several different types of ceramics have been successfully implanted in a variety of recipients. Some are ceramic composites done in our lab (8,23). Composites formed from ceramic plus other materials have been developed in attempts to get better products. Among these are ceramics composed of aluminum, calcium, and phosphorous oxides and referred to as ALCAP. They combine all of the best features of previous aluminum and calcium phosphate ceramics. ALCAP ceramics implants are unique in that they provide a multipurpose crystallographic system where one portion of the implant can be resorbed more rapidly than the others. Phases involving calcium and phosphorus are readily resorbed while other phases containing aluminates are resorbed less rapidly and remain to act as a stable framework for the development of new bone growth (9,17,18). SEM and x-ray analysis can detect these changes (9).

Many bone and implant studies have used various techniques in the analysis of bone growth and repair yet there are no known reports employing the approach used in this investigation. Keller et al. (16) used histomorphometric analyses to quantitatively determine the patterns of bony ingrowth into porous dental implants in the mandibles of rhesus monkeys. These authors point out in their discussion that other methods of assessment such as biochemical, histochemical or ultrastructural analyses are necessary to compare their results to normal bone. X-ray analysis

supplies biochemical data in situ thus combining biochemical and histochemical analysis. SEM provides the ultrastructural information. Our multiple labeling method increases the sensitivity of histomorphometry and gives a visual record of the timing of bone growth changes.

The labeling method used in this study is based upon the basic tetracycline labeling techniques explained by Milch et al. (22) and Frost (10). A label is administered twice with a known interval of time between doses. The labeling material is taken into the bone as the bone is mineralized. The tetracycline is incorporated into the bone mineral matrix and can be detected with ultraviolet (u.v.) light. It marks the leading edge or calcification front with a fluorescent band. The bone continues to grow during the time between labels so the second dose results in a second band separated from the first. The distance between these lines can be measured in order to determine the amount of bone growth during the time between doses. This information can be used to calculate bone growth rates and other dynamic data.

The multiple labeling technique can use a variety of compounds or fluorochromes in addition to tetracycline thereby producing bands of contrasting color. This is a useful technical refinement of earlier methods (1). Labeling with this multiple fluorochrome technique has several advantages. A persistent problem with double tetracycline labels is interpretation of ambiguous sites where only one dose results in a label. With different colors, it is easier to decide which label is missing (25). Colored bands can also be more readily separated in instances where the lines are close together. The multiple labels improve photographic recordings that show ossification processes that occur in bone. Different colors allow key events in an experimental protocol to be tied to a certain color for accurate identification of changes (7).

Photographic records and accurate timing information allow investigators to clearly demonstrate the ingrowth of bone into implants. They can also determine the dynamics for bone growth and repair.

Just as a truly interdisciplinary approach should be used for the analysis of biomaterials in the scientific community (2), a similar collaboration should exist throughout Armstrong Laboratories. Thus, the primary objective of this study was to develop a comprehensive approach for analyses of bone responses. Such an approach could then be developed into a standard procedure for biodynamic and toxicity studies involving bone. A second major objective was obtaining a greater understanding of osteogenic processes in order to better detect and explain effects of toxic agents or mechanical stresses.

Meeting these objectives would go beyond development of a testing platform for the different techniques and also serve as a springboard for an expanded list of projects. The techniques can be used to add to the knowledge of bone biodynamics under many conditions such as mechanical stress. Essentially most any study dealing with changes in bone could benefit from the different perspectives used in this study.

As this study began, there was a research project in progress at the Toxicology Division to examine bones (femurs) from rats exposed to Halocarbon 27S, a hydraulic fluid used by the Navy. The study involved using SEM and x-ray analysis to measure Ca/P ratios in bone to determine if the bone is altered by accumulation of fluorine from hydraulic fluid. The additional techniques used in this bone analysis procedure were designed to assist in studies just like this Halocarbon study. It might also be valuable to incorporate multiple pulse labeling and histomorphometry into other toxicity studies such as for PolyCTFE, an Air Force hydraulic fluid similar to the Navy's, that was proposed for use in the Advanced Tactical Fighter and Advanced Tactical Bomber.

One of the most exciting new theories in bone research is the proposal of "on - off" states for bone remodelling described by Hori et al (14). Results from this study can provide information allowing our laboratory to contribute to the validation process for this theory. If it is validated, this "on - off" state idea might help explain data obtained from mechanical testing. Triggering a change in state (on or off) with a mechanical load could result in changes in total bone formation and a net loss or gain in total bone mass.

Additionally, the methods described here could provide insight into the development of improved ways to handle and store tissue. It is common for bones to be maintained in low temperature freezers after necropsy until later transferal to alcohol for fixation. In many cases, partial defrost and thaw have occurred before processing begins. This can cause deleterious effects on the mechanical strength of bone (12). By using quick freezing in liquid nitrogen and almost immediate alcohol fixation, a comparison can be made with similar samples stored in freezers. If differences are found, this could lead to changes in normal storage standards.

#### METHODS

Twelve rhesus monkeys (*Macaca mulatta*), approximately 11 years old were used for this study. Since older humans suffer a proportionally greater number of bone problems and receive implants, an older population of research animals was considered appropriate for use in this study.

Each monkey was fully anesthetized and implanted, under aseptic conditions, with two ALCAP implants, one in the right femur and the other on the left side of the fourth lumbar vertebrae. Bone cores were removed from each site in order to implant the ALCAP. Cylinders of bone from the right femur were then used as autografts. They were implanted in the left femurs to provide

additional sites for examining bone repair in compact bone. Each cylinder of bone removed from the vertebrae was implanted into the right side of the adjacent third lumbar vertebrae to act as a sham control. It provided a site to examine repair in trabecular bone. This procedure is representative of current methods used in bone replacement surgeries. Pieces of bone are removed from separate sites within the body or taken from "bone banks" and used to repair bone at the injury sites. The use of composites could help avoid any problems with "tainted" bone.

Bone labelling compounds were administered at the times shown in Figure 1. The labelling sequence consisted of an alternating series of fluorochromes: tetracycline, xylenol orange and dicarbomethylaminomethyl fluorescein (DCAF). The initial labelling of bone was done two weeks prior to the surgical placement of the implants in order to demonstrate normal bone forming activity (baseline). Four monkeys each were euthanized at 1, 4 and 8 months after implantation (Figure 1). Normal primate bone turnover rates were considered in determining the length of time the animals were allowed to remain on study. Necropsy samples, including implants and surrounding bone, were retrieved and quickly frozen in liquid nitrogen.

Each implant sample was then divided into two sections by freeze fracturing. One section was transferred directly to 70% ethyl alcohol and processed for histomorphometry (HM) using standard histological procedures. The remaining section was stored in a freezer as was normally done under current operating procedures. Immediate transfer from the frozen state to alcohol fixation represented an attempt to alleviate any adverse effects on the bone specimen as is reported to occur from storage in the frozen state (12).

LABELS	L1	L2	L3	L4	L5	L6	L7	L8	L9	
MONTHS	B	0	1	2	3	4	5	6	7	8
		I	S			S				S
NUMBER OF ANIMALS:			4			4				4

B = Baseline, L = Labelling, I = Implantation, S = Sacrifice

Figure 1. Diagram of time points for experimental work

A Polycut sledge microtome, capable of cutting thin sections (less than 20 microns) of both bone and implant material, was used for this work effort. The microtome provided sections that were superior to those made previously with cutting and grinding procedures. A selected number of sections were prepared for SEM preparation. These sections were freeze-dried, mounted on a stub, sputter coated with platinum and placed in a scanning electron microscope (Model 1000B, Amray Inc., Bedford, MA) for photography and x-ray analysis.

## RESULTS

No perceivable differences were observed between samples fixed directly in alcohol after quick freezing and those stored in the freezer. It was difficult to compare them, however, because they could not be allowed to thaw. No biomechanical testing was performed to determine if any changes occurred in strength characteristics.

Histological evaluation of bone sections from animals sacrificed at 1, 4, and 8 months showed no measurable bone growth around the implant. Both sections stained with Villanueva osteochrome bone stain and unstained sections were used for the

evaluation. Gaps remained where bone had been removed surgically. There was no new tissue growing in from surrounding areas and filling the gaps or bridging over them. The unstained sections, viewed with UV light, showed very little bone growth activity in the bone adjacent to the implant site. The ALCAP ceramic had not been removed and could still be seen in the defect.

These results indicate that repair of the defect was not successful. This finding may be due to the age of the animals. Bone repair slows later in life. In time, bone growth may have eventually begun to fill in the areas around the implants.

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